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LIPID COMPOSITION OF *AZOTOBACTER VINELANDII* IN WHICH THE INTERNAL MEMBRANE NETWORK IS INDUCED OR REPRESSED

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SUMMARY

A vast internal membranous network in addition to the cytoplasmic membrane is induced in *Azotobacter vinelandii* simultaneously with the induction of the N_2-fixing enzymes. This membrane network is absent in *Azotobacter* growing with a fixed nitrogen source, *e.g.*, ammonia. This report compares the lipid composition of *Azotobacter* grown under internal membrane-induced and -repressed conditions. Although the total lipid content of the two types of cells is identical, cells possessing internal membranes contain (1) 30% more total phospholipid, (2) 50% more coenzyme Q, (3) 80% less neutral lipid and (4) 50% less anionic phospholipid (*e.g.* phosphatidylglycerol). The increased phospholipid (mostly amphoteric phosphatidylethanolamine) and coenzyme Q content of nitrogenase-induced cells correlate with greater respiratory activity, which may serve to protect O_2-labile nitrogenases.

INTRODUCTION

In addition to the cytoplasmic membrane, the N_2-fixing bacterium, *Azotobacter vinelandii* may contain an extensive internal membranous network[1,2]. The presence of the internal membrane network is directly related to the nitrogen source for growth; (a) *A. vinelandii* synthesizes great quantities of internal membranes when the nitrogenases are induced, *i.e.* when the bacteria are grown with air as the sole source of nitrogen and (b) the internal membranous network is absent when the formation of the nitrogenases is repressed, *i.e.* when the *Azotobacter* are grown with amino acids or ammonia[3]. Most likely, one of the major functions of these membranes is to maintain an optimal E_h within the cell[4–6]; the nitrogenases which are induced simultaneously with the internal membranes, are extremely labile to O_2. This report compares the content and types of lipid synthesized by the *Azotobacter* cultivated under conditions in which the internal membrane network is fully induced or repressed.

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MATERIALS AND METHODS

Organism and cultivation

Azotobacter vinelandii strain OP, a slime-free mutant was used in this study. 40-l cultures were grown in a Fermacel Fermentor Model F-30 at 30–32 °C in a modified Burk's nitrogen-free medium[3]. Fixed nitrogen as $(NH_4)_2SO_4$ (0.25 %) was added to repress the formation of the nitrogenases. 1 Vol. of the exponentially growing culture was poured over 1–2 vol. of crushed ice to halt metabolism abruptly.

Partition of lipid

Exponentially growing cells were washed with saline solution; the total lipid content was extracted from wet cells with chloroform–methanol (2:1, v/v) and washed by the procedure of Folch *et al.*[7]. The lipid was partitioned over a column of 100 mesh silicic acid by elution with 8 column volumes of solvents of increasing polarity according to the schedule shown in Table I[8,9].

Analysis of partitioned lipids

The partitioned fractions were further separated and analyzed by thin-layer chromatography over silica gel G using a solvent system of chloroform–methanol–water (65:25:4, by vol.)[10]. The diverse lipid compounds, primary amines and organic phosphates were detected by successive exposure of the chromatographic plates to I_2 vapor, ninhydrin spray and molybdate–acid spray[11]. Other functional groups were identified by infrared spectroscopy; Fractions 1–4 were spread as an oily film on transparent discs of NaCl. Fractions 5 and 6 were not entirely soluble in chloroform; hence were ground into KBr (approximately 1 mg per 200 mg KBr) and pressed into transparent discs.

Fraction 2 was estimated for coenzyme Q by reduction with $NaBH_4$ of the peak at 275 nm ($E^{1\%}_{1\,cm} = 163$)[9,12]. The primary amine content of phospholipid was calculated by the colorimetric measurement with trinitrobenzene sulfonic acid[13]. The phosphorous content of phospholipid was determined colorimetrically after $HClO_4$ digestion of organic phosphates as a molybdate–acid complex[14].

The phospholipid samples were further separated into amphoteric (neutral) phosphatidylethanolamine and anionic phospholipid by ion-exchange chromatography with DEAE-cellulose[15]. The eluents from chloroform–methanol (7:1 and 7:3, v/v) were designated amphoteric and contained predominately phosphatidylethanolamine ($R_F = 0.40$) by thin-layer chromatography. The anionic phospholipids were eluted with 1% conc. NH_4OH in chloroform–methanol (4:1, v/v) and showed spots corresponding to reference phosphatidic acid ($R_F = 0.9$ tailing spot) phosphatidylglycerol ($R_F = 0.50$), and cardiolipin ($R_F = 0.75$) compounds on thin-layer chromatography.

Fatty acid analysis

The neutral lipid fractions (pooled Fractions 1–3) and phospholipid (pooled Fractions 4–6) were saponified, acidified and subsequently extracted with diethyl ether to obtain their fatty acids. The fatty acids were then esterified with diazomethane and separated by gas–liquid chromatography. A 6 ft × 0.25 inch column of 20% diethyleneglycol succinate polyester on Chromosorb W was used to separate the methyl esters at 192 °C with He carrier (approximately 130 ml/min). The quantitative estimate of each peak was determined by the method of Carroll[16].

RESULTS

Total lipid and neutral lipids

The extractable lipid (total lipid) from exponentially growing cells rich in internal membranes, *e.g. Azotobacter* grown with air as the source of nitrogen, was compared with the lipid from an identical aliquot by wet weight of cells grown with NH_3 (Table VII, Part I). The amount of lipid per unit of wet cellular mass was identical. The total lipid was partitioned over silicic acid to separate the lipids into six fractions (Table I); the data listed as per cent of total recovered lipid are presented in Table II. A 6-fold greater quantity of neutral lipids (Fractions 1–3) containing a mixture of long chain hydrocarbons, coenzyme Q, fatty esters (*e.g.* diglycerides), fatty acids and yellow pigments was synthesized by cells grown on NH_3 than with atmospheric N_2. The air (N_2)-grown cells, which possess the greater quantity of internal membranes contained about a 30% higher phospholipid content (Fractions 4 and 5), or approximately 90% of its total lipid.

TABLE I

ELUTION SCHEDULE OF SILICIC ACID CHROMATOGRAPHY[8,9] OF THE LIPID OF *A. vinelandii*

Fraction	*Eluting solvent*	*Components eluted*
1	Light petroleum	Hydrocarbons
2	Light petroleum–10% diethyl ether	Coenzyme Q, fatty esters, fatty acids
3	100% diethyl ether	Polar fatty acids, free fatty acids, pigment
4	Chloroform–25% methanol	Phosphatidylglycerol, phosphatidic acids, mostly phosphatidylethanolamine
5	Chloroform–50% methanol	Mostly phosphatidylethanolamine
6	100% methanol	Lysophospholipids, highly polar phospholipids, contaminating amino acids, other polar compounds, and pigments

TABLE II

SILICIC ACID CHROMATOGRAPHY OF LIPIDS FROM *A. vinelandii* CELLS GROWN WITH AIR AND NH_3 AS NITROGEN SOURCES

Nitrogen source for growth	*% of total recovered lipid*					
	Designated fractions with (eluting solvent):*					
	1	*2*	*3*	*4*	*5*	*6*
Air (N_2)	1.4	3.1	0.9	74.0	16.6	4.0
$(NH_4)_2SO_4$	1.6	26.5	2.7	56.3	10.7	2.2

* Eight column-volumes of eluting solvent were passed over silicic acid stepwise with increasing polarity: (1) light petroleum, (2) 10% diethyl ether in light petroleum, (3) 100% diethyl ether, (4) 25% methanol in chloroform, (5) 50% methanol in chloroform, and (6) 100% methanol.

The six fractions described in Table II were surveyed further by infrared spectroscopy and thin-layer chromatography to dermine the lipid classes and characteristic functional groups. The infrared spectrum of Fraction 1 (Figs 1A and 1B) from both air (N_2)- and NH_3-grown cells suggests a long-chain alkane with branched

methyl groups (*e.g.* polyisoprenoid). Fraction 2 from the air (N_2)-grown cells showed intense absorption peaks for carbonyls (1740 and 1710 cm^{-1}) and sharp absorptions for aromatic quinones (1650 and 1610 cm^{-1}) thereby suggesting a content of fatty acids, fatty esters and coenzyme Q. In contrast, Fraction 2 from the NH_3-grown cells (Fig. 1B) showed weak quinone absorption peaks and more prominent carbonyl and ester double bond (3020 cm^{-1}) peaks suggesting a relative increase of fatty acids, fatty esters and unsaturated compounds.

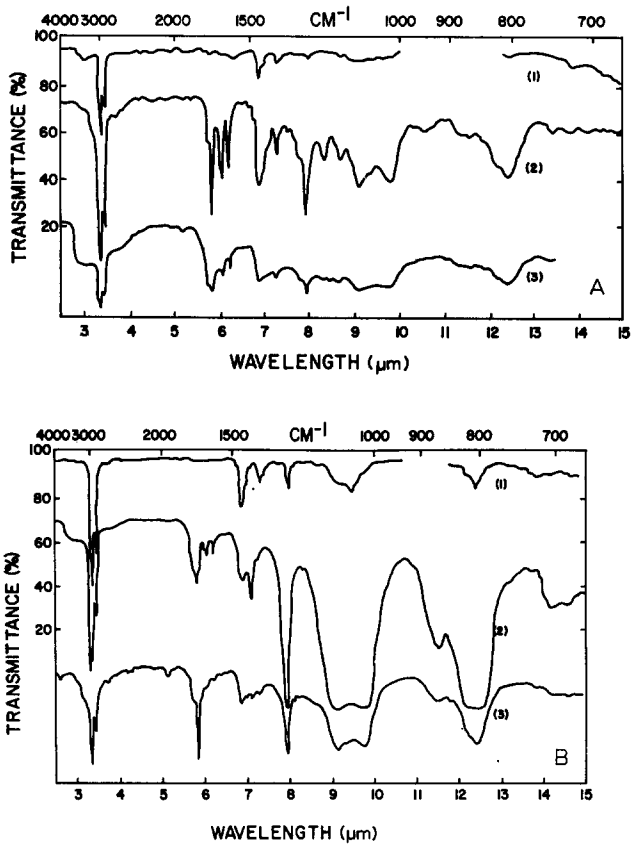

Fig. 1. (A) Infrared spectra of silicic acid chromatographed Fractions 1, 2 and 3 from the lipid of *Azotobacter vinelandii* grown with air as nitrogen source. (B) Infrared spectra of silicic acid chromatographed Fractions 1, 2 and 3 from the lipid of *Azotobacter vinelandii* grown with NH_3 as nitrogen source.

Thin-layer chromatography of the "neutral" Fractions, 1–3, uniformly yielded 4 spots. In a solvent system of light petroleum–methanol–water (70:30:10, by vol.), the R_F values over silica gel G consistently found were 0.20, 0.30 (tailing), 0.75, and 0.85. The first two spots are probably due to free fatty acids and esters, respectively. The spots of R_F 0.75 and 0.85 were pigmented; a reference coenzyme Q compound isolated from *Azotobacter* also migrated with an $R_F = 0.85$. Although the neutral lipid content of air (N_2)-grown cells is one sixth that of the NH_3-grown cells, the specific coenzyme Q concentration (Table III) was 1.5 times higher than that of the

TABLE III

YIELD OF TOTAL LIPID, COENZYME Q, AND AMPHOTERIC PHOSPHOLIPID (N/P = 1) FROM CELLS OF *A. vinelandii* GROWN WITH AIR AND NH_3 AS NITROGEN SOURCES

The lipids were chromatographed as shown in Table II. Total lipid was extracted from the centrifuged wet cells with chloroform–methanol (2:1, v/v). The coenzyme Q fraction was eluted from silicic acid with 10% diethyl ether in light petroleum (Fraction 2); and the phospholipid was eluted with methanol (combined Fractions 4, 5, and 6).

Nitrogen source for growth	*Total lipid (% wet cells)*	*Coenzyme Q (% total lipid)*	*Phospholipid (N/P ratio)*
Air (N_2)	2.82	1.04	1.19
$(NH_4)_2SO_4$	2.79	0.71	0.75

NH_3-grown cells. The larger proportion of neutral lipid in NH_3-grown cells is probably free fatty acids and diglyceride esters.

Phospholipids

The infrared spectra of both Fractions 4 show the typical phospholipid absorption frequencies[9]. Fractions 5 and 6 were similar to Fraction 4 except for the increase in infrared absorptions at the polar hydroxy and/or primary amines.

The thin-layer chromatography of pooled phospholipid Fractions 4, 5 and 6 in a solvent system of chloroform–methanol–water (62:25:4, by vol.) yielded 6 or 7 spots of compounds containing organic phosphate. Four consistent phospholipid spots migrated with R_F values of 0.18, 0.40, 0.50 and 0.75. The R_F values correspond to authentic samples of phosphatidylserine, phosphatidylethanolamine, phosphatidylglycerol and cardiolipin, respectively. The two former spots (R_F = 0.18 and 0.40) were ninhydrin-positive as expected. The N/P analysis of the pooled phospholipid indicates that NH_3-grown cells produce a significantly higher concentration of anionic phospholipid (higher phosphorous content with N/P of 0.75) than do air (N_2)-grown cells.

Anionic phospholipid

Since the phospholipid of NH_3-grown cells showed a high phosphorous content, pooled Fractions 4–6 were fractionated further by DEAE-cellulose to separate amphoteric phospholipid (neutral phosphatidylethanolamine) from anionic phospholipid (phosphatidylglycerol, phosphatidic acid and cardiolipin). NH_3-grown cells contain twice the proportion of anionic-type phospholipid (14%) than that produced by air (N_2)-grown cells (7%) (Table IV). However, both types of cells contained predominantly amphoteric phosphatidylethanolamine.

Fatty acid content

The fatty acids synthesized during growth with the two nitrogen sources were analysed from the neutral fraction (fatty acids and esters from Fractions 1–3) and the phospholipid fraction (fatty esters from Fractions 4–6) containing phosphorous compounds.

According to the data presented in Table V, the neutral lipid of the air (N_2)-grown cells contained approximately twice the saturated C_{16} fatty acid complement

TABLE IV

AMPHOTERIC AND ANIONIC PHOSPHOLIPIDS SEPARATED BY DEAE-CELLULOSE FROM CELLS GROWN WITH AIR AND NH_3 AS NITROGEN SOURCES

The procedure of Rouser *et al.*[15] was used to separate the phospholipid into amphoteric phosphatidylethanolamine (neutral) and anionic phosphatidylglycerol (acidic) fractions. Total phosphorus recovered by the colorimetric assay was about 110% of the lipids placed on the DEAE-cellulose column.

Nitrogen source for growth	*Phospholipid type separated (% of total phosphorus)*	
	Amphoteric	*Anionic*
Air (N_2)	96	7
$(NH_4)_2SO_4$	89	14

TABLE V

GAS–LIQUID CHROMATOGRAPHY OF FATTY ACIDS OF THE NEUTRAL LIPID (FRACTIONS 1, 2, AND 3) FROM CELLS GROWN IN AIR AND NH_3

Methyl ester of fatty acids	*Mole % of each fatty acid component*	
	Air (N_2) grown	*$(NH_4)_2SO_4$ grown*
C_{10}	0	0
C_{12}	Trace	0
C_{14}	6.4	3.9
C_{16}	28.4	15.9
ΔC_{16}	50.8	73.8
C_{18}	6.6	Trace
ΔC_{18}	7.8	6.4
Mole % unsaturation	59	80

TABLE VI

GAS–LIQUID CHROMATOGRAPHY OF FATTY ACIDS OF THE PHOSPHOLIPID (FRACTIONS 4, 5, AND 6) FROM CELLS GROWN IN AIR AND NH_3

Methyl ester of fatty acids	*Mole % of each fatty acid component*	
	Air (N_2) grown	*$(NH_4)_2SO_4$ grown*
C_{12}	0	0
C_{14}	5.0	4.1
C_{16}	27.7	26.7
ΔC_{16}	51.2	47.0
C_{18}	0	0
ΔC_{18}	16.1	22.2
Mole % unsaturation	67	69

and two-thirds the unsaturated C_{16} fatty acid content of their NH_3-grown cells counterpart. Little or no saturated C_{18} fatty acid was produced by the NH_3-grown cells. The air (N_2)-grown cells contained a lesser degree of unsaturated fatty acids, three-fourths that contained in the NH_3-grown cells. The neutral fraction also contained shorter-chained fatty acids of C_{12} to C_{14} (4–6 mole % of the total fatty acids).

On the other hand, the chain length of the fatty esters from the phospholipid fraction (Table VI) was uniformly between C_{14} to C_{18}, mainly hexadecanoic acids, with no evidence of the "stearate" peak. The unsaturated fatty acids from the phospholipid fractions of both types of cells were similar (68%).

DISCUSSION

The most striking difference in the lipid content of air (N_2) induced and NH_3 repressed *Azotobacter* is in the phospholipid content. 90–94% of the extracted lipid of the air (N_2)-grown cells is phospholipid, mainly phosphatidylethanolamine; whereas about 67% of the lipids of the NH_3-grown cells was phospholipid. The phospholipids of the air (N_2)-grown cells contain more amphoteric groups than do the NH_3-grown counterpart. Conversely, NH_3-grown cells contain twice the proportion of anionic lipid (phosphatidylglycerol) than do the former. The fatty acid components of the phospholipids in cells grown with either nitrogen source are similar and contained mainly hexadecanoic acids. Even though the major part of the neutral lipid of the nitrogenase-induced *Azotobacter* was only 3% of the total lipid compared with 26.5% for the nitrogenases-repressed *Azotobacter*, the former contained 1.5 times more coenzyme Q. This probably directly reflects the increased respiratory activity (see summary, Table VII).

TABLE VII

SUMMARY OF LIPID CONTENT OF *A. vinelandii* GROWN IN AIR AND NH_3

Lipid component	*Nitrogen source for growth*	
	Air (N_2)	$(NH_4)_2SO_4$
I. Wet wt of cells extracted (g)	30.62	26.62
Total lipid extracted (g)	0.862	0.742
Total lipid extracted (% of wet wt)	2.82	2.79
II. Neutral lipids (Fractions 1–3)		
% of total chromatographed lipids	5.4	30.8
(1) Free fatty acids and diglycerides	2.0	25.0
(2) Coenzyme Q (% of total lipid)	1.04	0.71
Coenzyme Q (% of neutral lipid)	(19.3)	(2.3)
(3) Fatty acid composition (mole %)		
C_{10}–C_{12} acids	Trace	0
Unsaturation of C_{14}–C_{16} acids	59	80
III. Phospholipids (Fractions 4–6)		
% of total chromatographed lipids	94.6	69.2
(1) N/P ratio	1.19	0.75
(2) Amphoteric phospholipid (mainly phosphatidylethanolamine) (% of total P)	96	89
(3) Anionic phospholipid (mixture of phosphatidylglycerol, cardiolipin and phosphatidic acid)	7	14
(4) Fatty acid composition (mole %)		
C_{10}–C_{12} acids	0	0
Unsaturation of C_{14}–C_{18} acids	67	69

From a resting cell suspension of air (N_2)-grown *A. vinelandii* disrupted by sonic treatment for 15 min, Jurtshuk and Schlech[17] isolate "R_3" electron transport particles (144_P120) which contain four of the six phospholipid fractions obtained from the intact cells. Both whole cells and the R_3 particles contain cardiolipin (I) and substantial quantities of phosphatidylethanolamine (II), the major phospholipid constituent of the *Azotobacter*, as well as yet undefined constituents III and IV. The "R_3" lack Fractions V and VI which are also unidentified. Since the electron transport particles are associated with the cell envelope[18,19] one might surmise that Fractions V and VI reside in distinct areas of the cell envelope and/or the internal membranes.

Our study is consistent with the idea that diglycerides and anionic phospholipids are, in general, precursors to amphoteric phospholipids such as phosphatidylethanolamine[20]. The induced air (N_2)-grown cells contained proportionally high concentrations of coenzyme Q and phosphatidylethanolamine; whereas the repressed cells (NH_3-grown) contained more neutral lipid (diglyceride, pigment, poly-β-hydroxybutyrate) and anionic phospholipids. Our findings clearly suggest that coenzyme Q and phosphatidylethanolamine are enriched in the internal membrane network. In preliminary experiments we have separated the internal membranes from osmotically-lysed *Azotobacter* by sucrose density gradient centrifugation[4]. Thus we should be able to assay the lipid content of the internal membranes directly.

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